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Distribution of vasoactive intestinal peptide and calcitonin gene-related peptide immunoreactive nerve fibers and binding sites in the hamster seminal vesicle during post-natal development

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Summary. The distribution of vasoactive intestinal peptide (VIP)- and calcitonin gene-related peptide (CGRP)-immunoreactive nerves and ^{125}I-labeled VIP- and CGRP-binding sites was studied in the hamster seminal vesicle of 12-, 30- and 60-day-old animals. In addition, the general innervation of the seminal vesicle was examined using the general neuronal marker synaptophysin.

Our results show that the densities of the overall (synaptophysin immunoreactive) and CGRP-immunoreactive innervation is constant during the post-natal development of the gland. However, a significant decrease in VIP-containing nerves is observed at the end of puberty. The autoradiographic study revealed that in 12-day-old animals, the epithelium presents VIP binding sites. However, in 30-day-old animals, VIP binding sites are observed in the epithelium of only a few clumps of acini. In 60-day-old animals, the gland is composed of acini with dilated lumina where VIP binding sites are not detected. In all groups studied the epithelium does not exhibit CGRP binding sites. The seminal vesicle muscle layer displays specific binding sites for both VIP and CGRP at all post-natal developmental times, but the density of VIP binding sites is higher in 12- than in 30- and 60-day-old animals.

Our results, showing the presence of specific VIP and CGRP binding sites during the development of the hamster seminal vesicle, suggest that these neuropeptides may be involved in the growth and differentiation of the gland.

Key words: Vasoactive intestinal peptide, Calcitonin gene-related peptide, Immunohistochemistry, Autoradiography, Seminal vesicle

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Introduction

The seminal vesicle is one of the male sex accessory glands whose structural and functional integrity is regulated through both hormonal and neuronal factors. Although the importance of hormonal control is a well established fact, the role of the autonomic nervous system is less clear in this gland.

It is now recognized that the nerve fibers supplying the seminal vesicle contain not only the classical neurotransmitters acetylcholine and noradrenaline, but also an array of different peptidergic transmitter candidates (Lange and Unger, 1990; Pinho et al., 1994, 1997; Stjernquist et al., 1983; Tainio, 1995). These neuropeptides may act as neurotransmitters, neuromodulators and/or trophic factors. In addition, neuropeptides may interact with androgens so leading to androgen stimulation of protein secretion in those glands (Kinghorn et al., 1987). The review of the developmental studies on neuropeptides in male sex accessory glands suggests that neuropeptides might be involved in gland growth and functional development during early stages of life (Properzi et al., 1992; Gosling and Dixon, 1994; Juarranz et al., 1994; Jen and Dixon, 1995).

Recent data from our laboratory obtained through immunohistochemical, autoradiographical and pharmacological experiments, showed that vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) may be involved in the regulation of secretion, smooth muscle tone and blood flow in the adult hamster (Afonso et al., 1996; Pinho et al., 1996). However, little is known about the ontogeny of VIP- and CGRP-containing nerves and their possible role in the development of seminal vesicle tissues.

In order to get further insight into the possible role of VIP and CGRP in the post-natal development of hamster seminal vesicle tissues we have employed quantitative immunohistochemical and receptor autoradiographic techniques to examine the distribution

of VIP- and CGRP-immunoreactive nerves and ^{125}I-labelled VIP- and CGRP-binding sites in the seminal vesicle of 12-, 30- and 60-day-old hamsters.

Materials and methods

Animals

Tissues were obtained from male hamsters (*Mesocricetus auratus*; Charles River Laboratories; n=12) killed by decapitation under ether anaesthesia. Animals were divided into three experimental age groups: I: 12-day-old (n=4), II: 30-day-old (n=4), and III: 60-day-old (n=4). The choice of these groups was based on studies showing that: I: the secretory vacuoles appear at the earliest at 12 days after birth; II: between 24 and 47 days there is an increase of about twofold in the intracellular secretory material, and III: the main features of the structure of the seminal vesicle secretory cells in adult hamsters are established between 48 and 62 days (for review see Mata, 1995).

Immunohistochemistry

Seminal vesicles were collected and fixed by immersion in Zamboni's fixative (Stefanini et al., 1967) for 16-24 h at 4 °C. Following thorough rinsing in phosphate buffered saline (PBS; pH 7.2) containing 15% sucrose (w/v) and 0.01% (w/v) sodium azide, tissues were processed as cryostat sections (10μm thick) for indirect immunofluorescence staining. Briefly, after pretreatment with a solution containing 0.2% Triton X-100 in PBS, for 30 min at room temperature, and impregnation with the dye pontamine sky blue, for 30 min, cryostat sections were incubated in diluted primary antiserum overnight at 4ºC. The preparations were then washed in PBS, incubated with biotinylated goat anti-rabbit IgG (1:200 dilution; Sigma Chemical Co, St Louis, Mo) for 1h at room temperature, rinsed in PBS and incubated with fluorescein isothiocyanate-labelled streptavidin (1:100 dilution; Sigma) for 1h at room temperature. The preparations were observed and photographed using an Olympus BH-2 microscope equipped with fluorescence epi-illumination.

Antisera

Primary antisera to calcitonin gene-related peptide (CGRP, code No. 1208, Hammersmith Hospital, London, UK), vasoactive intestinal peptide (VIP, code No. 652, Hammersmith Hospital, London, UK) and the general neuronal marker synaptophysin (SYN, code No. 1623, Dr. R. Jahn, Germany) were raised in rabbits and used at dilutions of 1:4000, 1:1600 and 1:3200, respectively. These antisera were characterized previously (Wharton et al., 1988; Gulbenkian et al., 1990). In control experiments no immunostaining was observed when the primary antiserum was omitted or replaced with pre-immune serum.

Receptor autoradiography

VIP

Unfixed cryostat sections (14 μm thick) were mounted onto poly-L-lysine-coated slides and air-dried for 2 h at 4 °C. Sections were preincubated for 30 min at room temperature in either a solution containing 0.005% polyethylenimine (v/v) in 50 mM Tris-HCl buffer (pH 7.4) or in the same solution containing also 1.25 μM human-VIP (Sigma), 0.1% bacitracin (w/v), and 1% bovine serum albumin (BSA; w/v), in order to uncouple bound endogenous VIP or to saturate VIP binding sites, respectively. Sections were then incubated for 60 min at room temperature in 50 mM Tris-HCl containing 0.125 nM ^{125}I-human-VIP (Amersham, sp. act. 2000 Ci/mmol), 0.1% bacitracin (w/v), and 1% BSA (w/v). Non-specific binding was assessed by incubating the sections with 0.125 nM ^{125}I-VIP in the presence of 1.25 μM unlabelled VIP. After washing in ice-cold Tris-HCl 50mM, and distilled water, the sections were fixed with 2.5% glutaraldehyde in PBS, for 30 min at room temperature, washed in PBS and air dried. Sections were covered by dipping in LM-1 nuclear emulsion (Amersham) and exposed at 4 °C for 28 h. Following development in Kodak D19 and fixation in 30% sodium thiosulphate (w/v), the sections were stained with Mayer's hemalum solution.

CGRP

For the labeling of CGRP binding sites, the same protocol as for VIP was employed, except that the incubations were performed with 0.125 nM ^{125}I-human α-CGRP (Amersham, sp. act. 2000 Ci/mmol) in the absence or in the presence of 1.25 μM human α-CGRP (Sigma) to measure total and nonspecific binding, respectively.

Quantitative analysis of immunostained nerves and autoradiograms

The analysis of the immunostained nerve areas and VIP/CGRP binding site densities was made using a JAVA video analysis software (Jandel Scientific, CA, USA) in combination with fluorescent and dark field microscopy (Olympus BH-2), respectively. After choosing a threshold level, so that maximum differentiation from background was obtained, the immunostained nerve areas and autoradiographic silver grain densities were quantified.

Immunostained nerves

In order to examine specifically the mucosal and muscular innervation, both the lumen and the external adventitia were excluded from each field analysed. Four cryostat sections collected at 100 μm intervals from one seminal vesicle from each animal were immunostained

Table 1. Total areas and total number of grains analyzed.

	AREA IN TOTAL BINDING	ARE IN NONSPECIFIC BINDING	GRAINS FOR TOTAL BINDING	GRAINS FOR NONSPECIFIC BINDING
VIP	$86.9x10^4\mu m^2$	$88.5x10^4\mu m^2$	18852	6096
CGRP	$75.5x10^4\mu m^2$	$77.3x10^4\mu m^2$	6440	2431

Values are sums from twenty-four autoradiograms (two per gland) from twelve separate animals.

Table 2. Density (%) of synaptophysin, VIP and CGRP immunoreactive nerve fibers in the muscle of the hamster seminal vesicles of 12-, 30- and 60-day-old animal (mean±SEM).

	SYNAPTOPHYSIN	VIP	CGRP
12-day-old	1.33±0.13	0.32±0.03*	0.12±0.03
30-day-old	1.37±0.20	0.28±0.05	0.10±0.02
60-day-old	1.30±0.08	0.25±0.02*	0.09±0.02

Mean values were derived from 12 sections of 4 seminal vesicles from 4 separate animals. *: significantly different between 12-day-old and 60-day-old animals.

for SYN, VIP and CGRP. For each group, the total areas of tissue analysed were $1.9x10^6\ \mu m^2$ for SYN, $1.8x10^6\ \mu m^2$ for VIP and $1.9x10^6\ \mu m^2$ for CGRP.

Autoradiograms

The total area of tissues and the total number of grains analysed in total binding and non-specific binding are presented in Table 1. The binding of ^{125}I-VIP/CGRP to epithelial and muscle layers was evaluated through the silver grain density over those gland compartments. Specific binding was assumed whenever the grain density obtained in total binding conditions was statistically different from that in non-specific binding situations.

Data analysis

Values were expressed as mean ± S.E.M. and the means were compared using the Student's t-test. Probability values of $p<0.05$ were considered significant.

Results

Histology

In 12-day-old animals acini were either solid or had a small lumen and the epithelium was pseudo-stratified (2b,d,f, 4b). In the seminal vesicle of 30-day-old animals the epithelium was convoluted, and acini were larger (3b,d, 4d). In the adult animals (60-day-old animals) the lumen of the acini was well developed with numerous and prominent folds (3f, 4f,h). In the three groups of animals, the acini were surrounded by a smooth muscle coat and an external connective tissue sheath.

SYN immunohistochemistry

In the seminal vesicle of all age groups, synaptophysin-immunoreactive nerve fibers were observed within the muscle coat running between smooth muscle cells, and in the lamina propria underlying the epithelium. Nerve bundles showing immunoreactivity for synaptophysin were identified in the interstitial connective tissue and external coat of the gland (Fig. 1a-c).

Quantitative analysis of the synaptophysin-immunoreactive innervation expressed as the percentage of stained nerve area showed similar densities in the glands of 12-, 30- and 60-day-old hamsters (Table 2).

VIP immunohistochemistry

VIP-immmunoreactive nerve fibers were detected in the seminal vesicle of the three age groups studied, being more numerous in the mucosal layer, just beneath the epithelium, than in the smooth muscle coat where they were sparse (Fig. 1d-f). VIP-positive nerve fibers were also present around blood vessels. Significant differences regarding the density of these nerve fibers in the seminal vesicle were only found between 12- and 60-day-old hamsters, corresponding to a decline in the density of VIP nerves with age (Table 2).

CGRP immunohistochemistry

CGRP immunoreactivity was mainly localized in nerve bundles running in the connective interstitium, in close vicinity to the smooth muscle layer. Scattered CGRP-positive nerve fibers were also found within the muscle layer, in the subepithelial connective tissue and in association with blood vessels (Fig. 1g-i). This pattern of distribution was similar in the three age groups studied. No significant differences were found between the density of CGRP-immunoreactive nerve fibers in the seminal vesicles of these groups (Table 2).

VIP binding sites

A different distribution pattern of labeling in the three ages group studied is shown in autoradiograms from total binding of ^{125}I-VIP to the seminal vesicle (Figs. 2, 3). On 12-day-old animals the acini were all labeled, although some acini labeled more intensely than others (Fig. 2a,b,e,f). In 30-day-old animals only a few acini were heavily labeled. The labeling depended

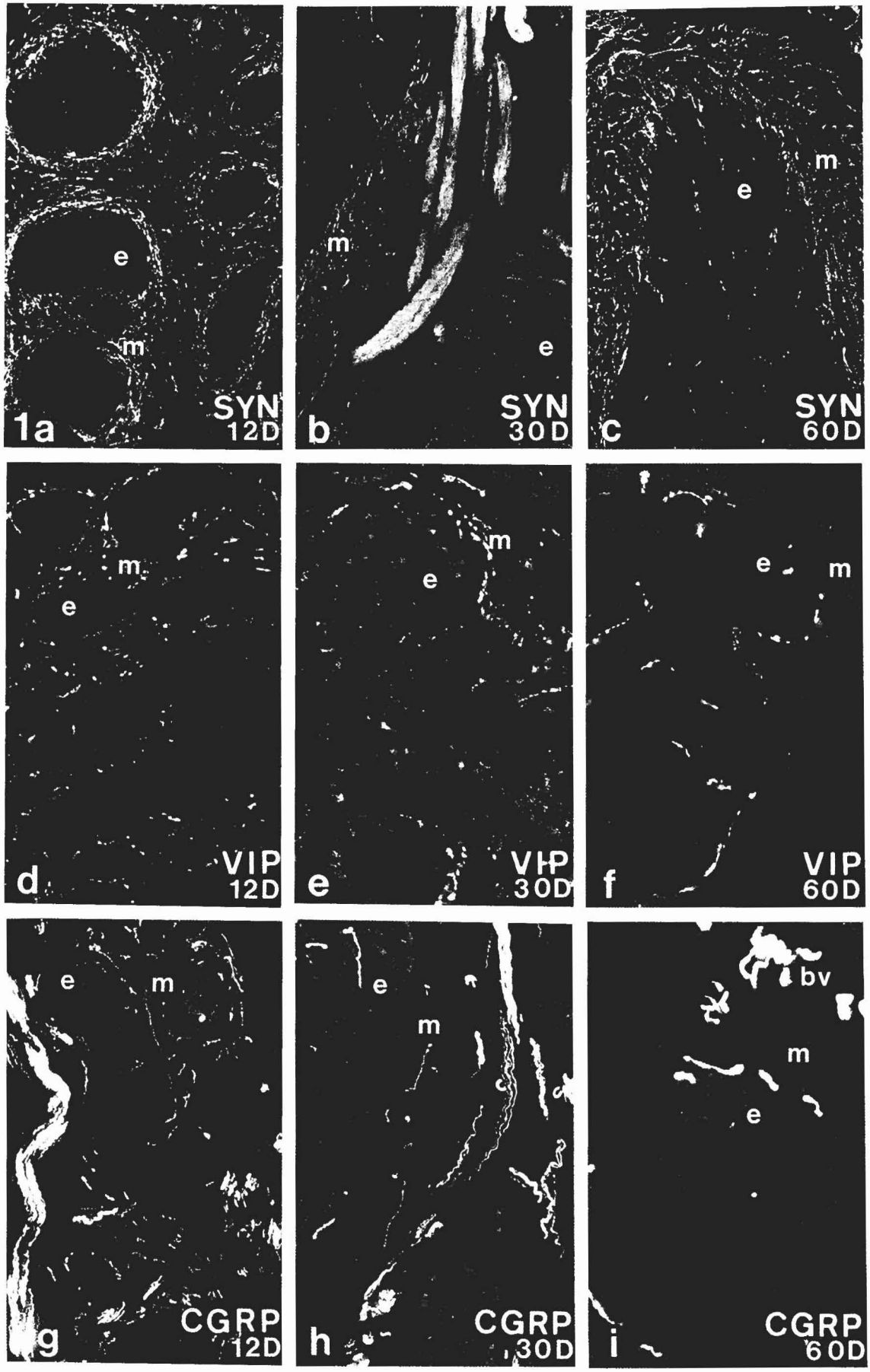

Fig. 1. Cryostat sections of seminal vesicles from 12- **(a,d,g)**, 30- **(b,e,h)** and 60-day-old hamsters **(c,f,i)**, immunostained for SYN (Fig. 1a-c), VIP (Fig. 1d-f) and CGRP (Fig. g-i). Numerous SYN-containing nerves are observed in association with the smooth muscle cells and with the epithelium (a-c). VIP-containing nerves are numerous beneath the epithelium, and relatively sparse in the smooth muscle coat (d-f). CGRP-containing nerves are localized close to the smooth muscle layer in nerve bundles. A few CGRP-containing nerve fibers are also found between the muscle cells, beneath the epithelium (g-i) and in association with blood vessels (1i). e: epithelium; m: muscle. a-c, g-i x 144; d-f: x 289

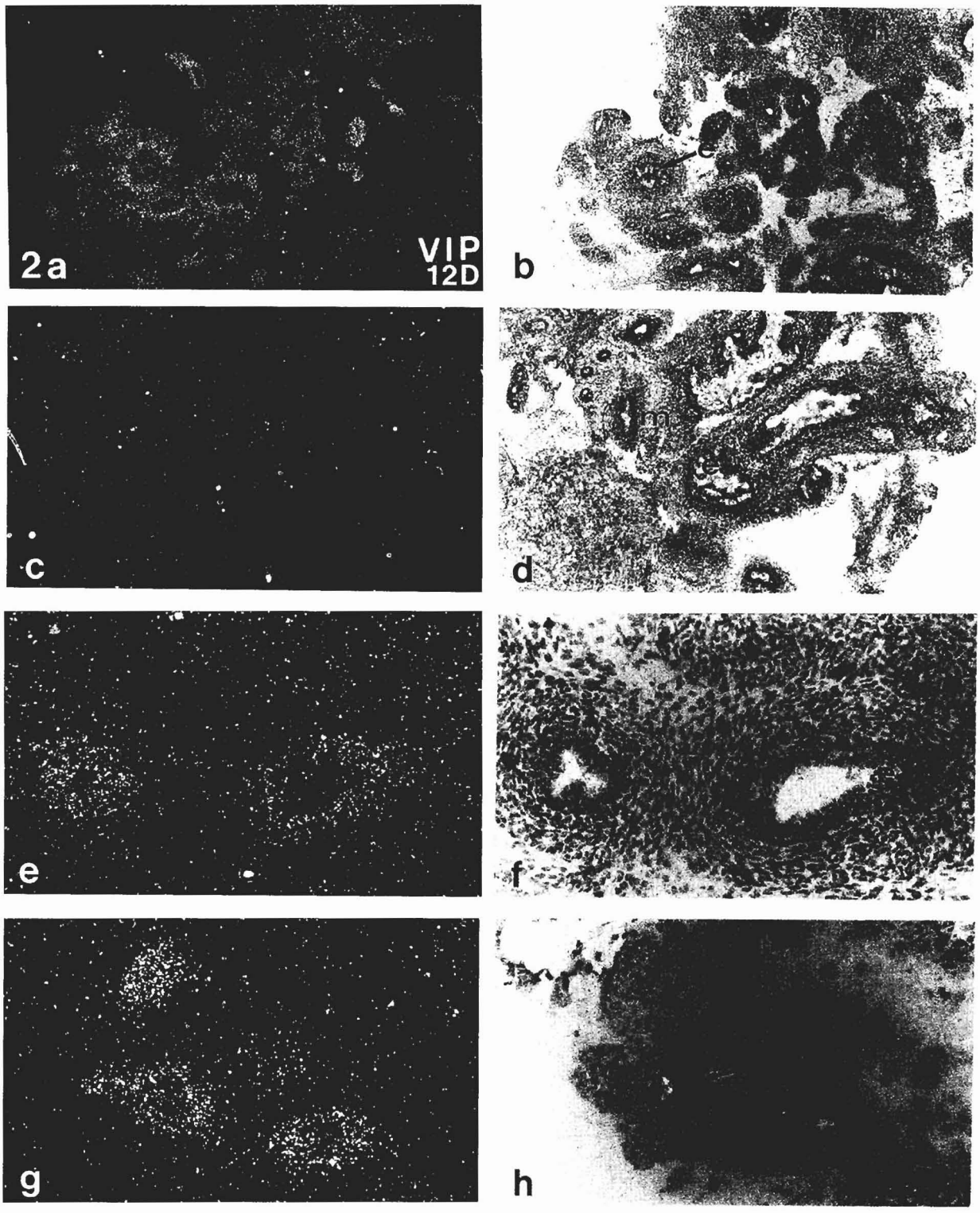

Fig. 2. Autoradiograms illustrating ^{125}I-VIP binding in hamster seminal vesicle of 12-day-old animals tissues in the absence (**a,b,e-h;** total binding) or in the presence (**c,d**; nonspecific binding) of unlabeled VIP. Dark-field (a,c,e,g) and bright-field (b, d, f, h) photographs of the same field are shown. In 12-day-old animals the epithelium presents binding sites. Some acini and muscular areas are intensely labeled. Heavily labeled blood vessels are observed in g-h. The muscle layer displays specific binding sites for ^{125}I-VIP. e: epithelium; m: muscle. a-d,x 41; e-h, x 213

neither on the size of the lumen nor on the degree of epithelium convolution (Fig. 3a,b). In the adult animals (60-day-old animals) the acini showed a low density of silver grains (Fig. 3e,f). On the muscular coat, an accumulation of silver grains was observed in the glands from all age groups studied. In 12-day-old animals it was observed that some muscular areas received a high density of silver grains (Fig. 2a,b). An intense labeling was also detected over the wall of numerous blood vessels at all post-natal developmental ages studied (Fig. 2g,h). Autoradiograms from non-specific binding presented a low and homogeneous labeling (Fig. 2c,d, 3c,d).

Quantitative assessment of autoradiographic grain density over seminal vesicle gland tissues showed that specific binding of ^{125}I-VIP was associated with all acini from 12-old-day animals. In 30-day-old animals, specific binding of ^{125}I-VIP was found associated with only a few clumps of acini. In 60-day-old animals, acini did not display specific binding sites (Table 3). The muscle layer from 12-, 30- and 60-day-old animals showed specific ^{125}I-VIP binding sites.

CGRP binding sites

Autoradiograms from total binding and nonspecific

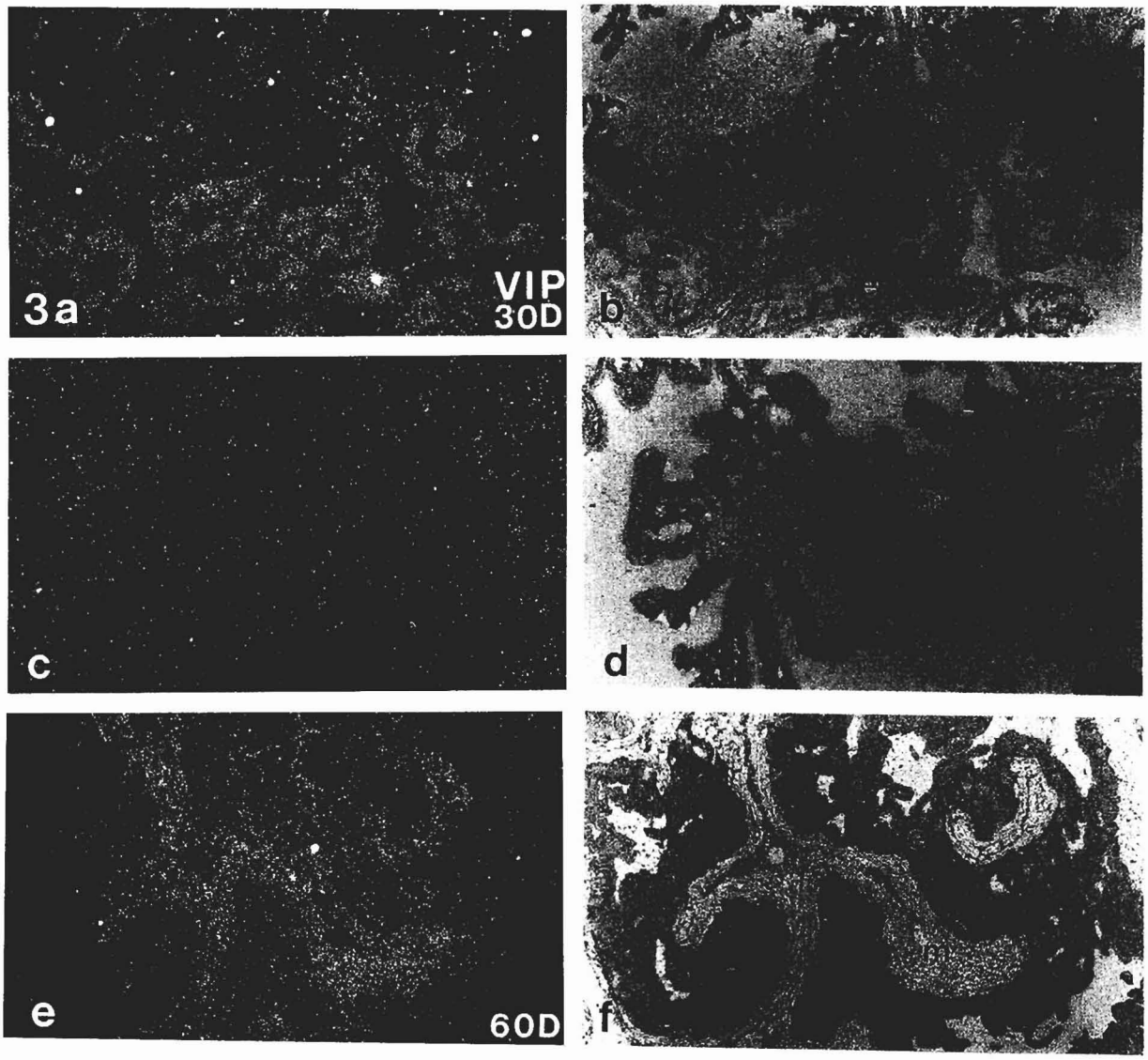

Fig. 3. Autoradiograms illustrating ^{125}I-VIP binding in hamster seminal vesicle of 30- **(a-d)** and 60-day-old animals **(e-f)** tissues in the absence (a,b, e,f; total binding) or in the presence (c,d; nonspecific binding) of unlabeled VIP. Dark-field (a,c,e) and bright-field (b,d,f) photographs of the same field are shown. In 30-day-old animals clumps of acini display specific binding sites for ^{125}I-VIP which are shown on a, b. In 60-day-old animals (e,f) ^{125}I-VIP binding sites are not observed. The muscle layer displays specific binding sites for ^{125}I-VIP at all times. e: epithelium; m: muscle. a,b,e,f, x 41; c,d, x 103

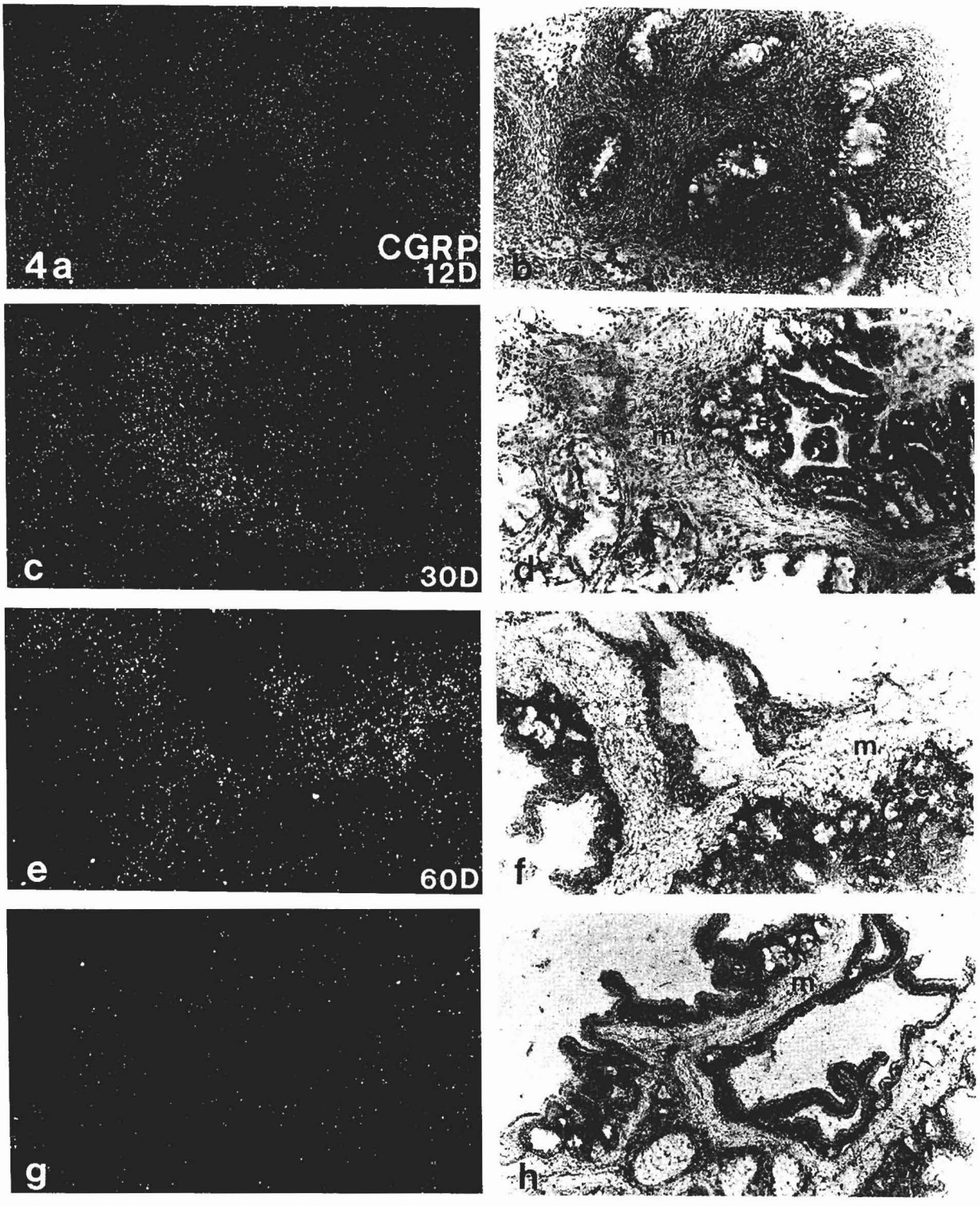

Fig. 4. Autoradiograms illustrating ^{125}I-CGRP binding in hamster seminal vesicle of 12- (**a,b**), 30- **(c,d)** and 60-day-old animals (**e-h)** tissues in the absence (a-f; total binding) or in the presence (g-h; nonspecific binding) of unlabeled VIP. Dark-field (a,c,e,g) and bright-field (b,d,f,h) photographs of the same field are shown. The seminal vesicle muscle layer presents specific binding sites for ^{125}I-CGRP, in all the 3 groups studied. e: epithelium; m: muscle. x 103

Table 3. Density of autoradiographic grains over cryostat sections of hamster seminal vesicle (mean±SEM/100μm^2) incubated with ^{125}I-VIP.

	TOTAL BINDING	NONSPECIFIC BINDING
12-day-old		
Epithelium	3.50±0.15*	0.76±0.04
Muscle layer	3.46±0.13*	0.60±0.05
30-day-old		
Epithelium (acini with labeling)	4.20±0.17*	0.84±0.05
Epithelium (acini without labeling)	0.74±0.05	0.67±0.05
Muscle layer	1.66±0.07*	0.70±0.04
60-day-old		
Epithelium	0.58±0.04	0.64±0.04
Muscle layer	1.49±0.06*	0.62±0.04

Mean values were obtained from four separate animals (one gland per animal; two autoradiograms per gland). *: significantly different from nonspecific binding ($p<0.001$).

Table 4. Density of autoradiographic grains over cryostat sections of hamster seminal vesicle (mean±SEM/100μm^2) incubated with ^{125}I-CGRP.

	TOTAL BINDING	NONSPECIFIC BINDING
12-day-old		
Epithelium	0.44±0.03	0.37±0.02
Muscle layer	1.61±0.09*	0.33±0.03
30-day-old		
Epithelium	0.26±0.03	0.29±0.03
Muscle layer	1.32±0.03*	0.34±0.04
60-day-old		
Epithelium	0.25±0.02	0.23±0.02
Muscle layer	1.28±0.07*	0.33±0.03

Mean values were obtained from four separate animals (one gland per animal; two autoradiograms per gland). * Significantly different from nonspecific binding ($p<0.001$).

binding of ^{125}I-CGRP to seminal vesicle sections are shown in Fig. 4. Autoradiograms from total binding experiments showed that the labeling pattern in the seminal vesicle of 12-, 30- and 60-day-old hamsters was similar (Fig. 4a-f). An accumulation of silver grains was mainly associated with the muscle layer. The density of silver grains over the muscle coat was significantly higher in total binding experiments than in autoradiograms from nonspecific binding where the labeling was low (Fig. 4g,h).

Quantitative assessment of autoradiographic grain density over seminal vesicle compartments is shown in Table 4. Specific binding of ^{125}I-CGRP was associated only with the muscle coat.

Discussion

The use of an antiserum to the general neuronal marker synaptophysin showed that the hamster seminal vesicle (HSV) is densely innervated at each post-natal developmental time analysed. Regarding the peptidergic innervation it was shown that while the density of the CGRP-immunoreactive nerves stays constant during the post-natal development of the gland, the density of VIP-containing nerves decreases significantly at the completion of puberty. The autoradiographic study revealed that the gland of 12-day-old hamsters displayed binding sites for both VIP and CGRP. It was also found that while CGRP binding sites are present only in the gland muscle coat and their density remains constant throughout post-natal development, VIP binding sites are expressed in different tissues of the seminal vesicle and their level of expression within those tissues varies significantly during the post-natal development of the gland.

VIP is presumed to be present in parasympathetic cholinergic neurons and CGRP in sensory nerve fibers supplying the mammalian male accessory sex glands (Lamano Carvalho et al., 1986; Chapple et al., 1991; Maggi, 1993; Pinho et al., 1994; Tainio, 1995). In addition, recent studies suggest that androgens selectively influence the morphology of different nerve populations in reproductive organs of the rat (Keast and Saunders, 1998). These works could explain our results, showing that CGRP-containing nerves remain constant throughout the post-natal development of the HSV, following the growth of the muscle during the development of the gland, in opposition to VIP-containing nerve fibers.

In the HSV, nerves containing VIP and CGRP are expressed in the early days of life (12 days after birth). In the human seminal vesicle, the autonomic innervation, except for the subepithelial VIPergic innervation, is already well established at 1 month postpartum (Gosling and Dixon, 1994). However, in the rat seminal vesicle, the peptidergic innervation appears in the genital system after birth, develops during post-natal life, and only reaches a full development between 20 and 38 days after birth (Properzi et al., 1992). These results suggest that the development of the peptidergic innervation varies depending on the species.

The presence of VIP binding sites in the wall of blood vessels associated with VIP immunoreactive nerve fibers at each of the ages studied, suggests a role of this neuropeptide in the regulation of HSV blood flow.

To our knowledge, this study characterizes for the first time the presence and distribution of VIP and CGRP binding sites during the development of the mammalian male accessory sex glands. In 12-day-old animals, all the acini display VIP binding sites, while in 30-day-old animals only a few acini are labeled. In

opposition, no specific binding sites are detected in acini from 60-day-old animals. It should be noted however, that when we consider two compartments in the epithelium of adult animals basal epithelium and apical epithelium, specific binding of ^{125}I-VIP is found to remain associated with the basal epithelium, as already demonstrated in our laboratory (Pinho et al., 1994). The decline of ^{125}I-VIP in the epithelium of the HSV during development can be related to the decrease of density of nerves positive to VIP from the glands of 12-day-old animals to 60-day-old animals. It has been shown in different systems, that the surrounding ligand concentration influences the number of available VIP receptors (Luis et al., 1986; Rosselin et al., 1988).

In the golden hamster, testosterone and DHT levels do not vary significantly until 36 days of age, then increase, showing a peak at 46-60 days of age, and finally decrease to the adult levels (Frungiere et al., 1996). The present study shows that VIP-specific binding sites in the HSV epithelium decrease during a period of development where testosterone levels are increasing, suggesting that VIP receptor expression is not a strictly androgen-dependent process. Pinho et al. (1996) reported that there was no significant decrease in ^{125}I-VIP binding sites in the muscular coat of the hamster seminal vesicle after medium-term (15 days) castration, whereas VIP nerves do not seem to be affected by castration. However, Juarranz et al. (1994) showed that ^{125}I-VIP binding, in membranes from the rat ventral prostate, follows a similar pattern to that of the circulating levels of testosterone during post-natal development. These results may reflect the use of either different organs or animal species or different methods. Reports have shown that ^{125}I-VIP-binding site levels in the rat prostate are regulated by androgens (Carmena et al., 1986, 1988). It could be speculated that these apparent contradictory conclusions reflect interactions between VIP and androgens that are not always permanent or exclusive, and that other factors may be associated.

The present results, showing that the expression of epithelial VIP-binding sites declines as the post-natal development of the HSV proceeds, suggest a possible physiological role for VIP in the epithelium of the HSV. The possibility that VIP is involved in the differentiation of the HSV epithelium is also supported by reports that suggest that VIP functions as a growth/differentiation factor for other epithelial cells, such as keratinocytes (Haegerstrand et al., 1989) and retinal pigment epithelial cells (Koh and Kane, 1992).

In the 3 groups studied ^{125}I-VIP- and ^{125}I-CGRP-binding sites are present in the muscle coat. The development of the stroma during the first month of life, when the levels of androgens are low (Frungieri et al., 1996), strongly suggests that besides androgens, other factors (including neuropeptides) are mediators of the differentiation and maintenance of the seminal vesicle. Earlier studies showed that CGRP and VIP modulate the development and proliferation of cells and CGRP was found to be mitogenic and to act as a differentiating factor (New and Mudge, 1986; Dennis-Donini, 1989; Haegerstrand et al., 1989, 1990; Koh and Kane, 1992; Pincus et al., 1990; Gressens et al., 1997). While VIP and CGRP receptors may be involved in these processes during the development, in the adult they may participate in the neural regulation of the gland secretion and contractility (Pinho et al., 1994; Afonso et al., 1996).

This study shows that VIP- and CGRP-positive nerve fibers, and ^{125}I-VIP- and ^{125}I-CGRP- binding sites are present in the seminal vesicle of 12-, 30- and 60-day-old HSV. This period coincides with the differentiation of the gland, suggesting that VIP and CGRP could be involved in the morphological and functional development of the seminal vesicle.

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